Minireview

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 269, No. 13, Issue of April 1, pp. 9397–9400, 1994

Glutathione-Ascorbic Acid Antioxidant System in Animals*

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There is continuing interest in cellular antioxidants and in the idea that certain diseases are produced (or made worse) by reactive oxygen species (*e.g.* oxygen free radicals, hydrogen peroxide) (1).[1] Cells have a number of mechanisms for dealing with the toxic effects of oxygen. One of the most important, perhaps the most important, is connected with the widely distributed tripeptide thiol glutathione (L-γ-glutamyl-L-cysteinyl-glycine; GSH). Indeed there is a strong evolutionary link between GSH and eukaryotic aerobic metabolism that is reflected in the function of GSH in protection against oxygen toxicity (2). GSH is not required in the diet of animals but is synthesized in virtually all animal cells[2] by the sequential actions of two enzymes: γ-glutamylcysteine synthetase and GSH synthetase. A useful approach to understanding the functions of a cellular component such as GSH is to remove it and to determine the consequences of its deletion. GSH deficiency can be produced *in vivo* by administering to animals (orally or by injection) a transition-state inhibitor of γ-glutamylcysteine synthetase, *e.g.* L-buthionine-(*SR*)-sulfoximine (BSO).[3] BSO (in phosphorylated form) binds tightly to the active site of γ-glutamylcysteine synthetase, thus inhibiting it irreversibly (3). Cellular GSH levels decrease after BSO is given because export of GSH continues in the absence of significant intracellular synthesis.

When such GSH deficiency is produced in newborn rats or guinea pigs, the animals develop multiorgan failure and die within a few days (4, 5). This result is directly related to the loss of an essential antioxidant system. The animals exhibit focal necrosis in liver, proximal tubular damage in kidney, and disruption of lamellar bodies in the lung; the newborns develop brain damage (6) and also cataracts (7). Cellular damage chiefly involves the mitochondria (which do not synthesize GSH but transport it from the cytosol), but also other structures, and affects both the water-soluble and the lipid phases of cells. The observed morbidity and mortality unequivocally attest to the major importance of the GSH antioxidant system for cell survival and function. Virtually no cellular defects are found after giving L-buthionine-(*R*)-sulfoximine, the diastereomer of L-buthionine-(*SR*)-sulfoximine that does not inhibit γ-glutamylcysteine synthetase. When the animals are given BSO plus GSH ester,[4] a cellular GSH delivery agent, tissue damage and mortality are greatly decreased, and in newborn rats, cataracts are almost entirely prevented in a dose-dependent manner (11).

The discovery that the lethal and other effects of GSH deficiency can be prevented by administration of ascorbate was made in the course of studies on newborn rats (4); later research on guinea pigs (12), which also do not synthesize ascorbate, and on adult mice (10, 13, 14), which do, has shown that there are significant interrelationships between GSH and ascorbate (15). Early studies suggested that dehydroascorbate reductase activity is present in animal tissues (16), and there is good evidence for its presence in plants (17). Borsook *et al.* (18) concluded that GSH is involved in this reaction in animal tissues, but another early pioneer, Guzman-Barron (19), dismissed the idea that this interaction is of major importance. Later studies suggested that animal tissue preparations can catalyze GSH-dependent reduction of dehydroascorbate (20–23), and Wells *et al.* (24) found that very highly purified preparations of protein disulfide isomerase and glutaredoxin can catalyze this reaction. The reaction also occurs nonenzymatically (18, 25) and may be driven by GSSG reductase. Many findings support the view that GSH, maintained in its reduced form by NADPH-dependent GSSG reductase, is a major player in cellular metabolism (26–29) and that GSH functions directly in the destruction of reactive oxygen species and as a substrate for GSH peroxidases (both selenium-containing and others), which catalyze the reduction of hydrogen peroxide and other peroxides. GSH also functions in the reduction of many cell components in addition to ascorbate.

Recent reports emphasize the potential significance of various antioxidant molecules, but reactions that are involved in recycling of these and in the production and maintenance of cellular reducing power need also to be considered. Cells normally produce substantial quantities of reactive oxygen species. Mitochondria are a major source of oxidants. The dramatic effects of GSH deficiency emphasize the importance of the GSH system, which supplies cells with a fairly constant level of GSH, in the millimolar range, that is needed to contain the large normal production of oxidants. After oxidation, an antioxidant might be regenerated by reduction, or it might be broken down and therefore need to be replaced. In the case of ascorbate, oxidation leads to dehydroascorbate, which, if not reduced, is irreversibly degraded. Although some ascorbate is degraded or excreted, only very small amounts of dietary ascorbate suffice to prevent scurvy in guinea pigs and humans. Administration of dehydroascorbate to guinea pigs can prevent scurvy. It is therefore evident that ascorbate is recycled *in vivo*; when large amounts of ascorbate are given, recycling may become less important. This minireview emphasizes relationships between GSH and ascorbate that have been studied in animals that can synthesize ascorbate (*e.g.* adult mouse) and those that cannot (*e.g.* newborn rat, guinea pig).

GSH Deficiency in Newborn Rats and Adult Guinea Pigs; Sparing of GSH by Ascorbate

When newborn rats are made GSH-deficient by administration of BSO, they develop cellular damage in liver, kidney, lung, and brain. They die within a few days. Death and tissue damage can be prevented by administration of ascorbate (but not of dehydroascorbate). GSH deficiency is accompanied by a marked decrease of tissue ascorbate levels. Thus, administration to newborn rats of BSO (6 mmol/kg/day for 3.5 days) led to a decrease of the ascorbate level from control levels of 2.51 ± 0.3 mmol/g to 0.42 ± 0.15 mmol/g in the liver, a decrease of about 80% (4). The corresponding values for total ascorbate (ascorbate + dehydroascorbate) were 2.60 ± 0.28 (control) and 1.14 ± 0.23 mmol/g, indicating markedly increased levels of dehydroascorbate. Similar values were obtained in other tissues. Normally very little dehydroascorbate is found in tissues. The tissue ascorbate levels of GSH-deficient animals were greatly increased by giving ascorbate, as expected, but surprisingly, giving ascorbate also led to higher GSH levels. The mitochondrial GSH levels were 2.7–6.0-fold higher in the tissues of newborn rats given BSO plus ascorbate as compared with those given only BSO. Thus,

* This minireview will be reprinted in the Minireview Compendium, which will be available in December, 1994. The research described here that was carried out in the author's laboratory was supported in part by National Institutes of Health Grant 2 R37 DK 12034 from the United States Public Health Service. Several general reviews on glutathione (3, 10, 26–29) and ascorbic acid (58, 59) have appeared and are cited in the text.

[1] This idea reflects the untoward effects of free radicals, which are of course formed and usefully involved in normal metabolism; they are also involved in the induction of certain genes and in protection against bacteria and viruses.

[2] GSH is also synthesized by many microorganisms and plants.

[3] The abbreviations used are: BSO, L-buthionine-(*SR*)-sulfoximine; LDL, low density lipoprotein.

[4] GSH mono(glycyl) esters (ethyl, isopropyl) have usually been used (8); recent studies indicate that GSH diethyl ester is more effective (9). The esters are converted to GSH intracellularly. GSH diethyl ester is about as effective as GSH monoethyl ester in mice and rats whose blood plasma exhibits high GSH diethyl ester α-esterase activity. In other animals (including humans), the diester is not split extracellularly and serves as an efficient cellular delivery agent for GSH monoester, which is formed intracellularly where it is converted to GSH over a period of time (9). Extracellular GSH itself, whether supplied orally or parenterally, is not effectively transported into most cells (10).

ascorbate spares GSH under these conditions. Treatment of newborn rats with only two doses of BSO (on the 2nd and 3rd days of life) led to cataracts, observed when the rats opened their eyes on days 14–16. The incidence of cataracts was 97% but was only 9% in animals given both BSO and ascorbate (2 mmol/kg/day).

Treatment of guinea pigs with BSO led to findings similar to those made on newborn rats (12). There was tissue damage and decreased tissue ascorbate levels, and the animals died within a few days. These effects were largely prevented by administration of ascorbate.

GSH Deficiency in Adult Mice; Sparing of GSH by Ascorbate

Sparing of GSH by ascorbate was also observed in studies on adult mice, but the findings are somewhat different because adult mice can synthesize ascorbate. Treatment of adult mice with BSO does not produce mortality and has no detectable effect on the cellular structure of liver and kidney because these tissues are protected by the presence of significant amounts of ascorbate. The hepatic level of ascorbate increases substantially early in GSH deficiency (14). Thus, 4 h after administration of BSO, the level of ascorbate in the liver doubles; thereafter, the ascorbate level decreases and that of dehydroascorbate increases. The level of ascorbate in the other tissues decreases and that of dehydroascorbate increases. These findings provide further evidence that GSH functions *in vivo* in the reduction of dehydroascorbate. Induction of hepatic ascorbate synthesis in GSH deficiency is reminiscent of the induction of ascorbate synthesis that occurs in the livers of rats after administration of certain drugs (30–32). The molecular mechanism by which GSH deficiency triggers ascorbate synthesis needs study. This mechanism is not active in newborn rats (1–5 days of age).

Although the liver and kidney are essentially unaffected by GSH deficiency in adult mice, this is not true for the lung, in which there is type 2 cell lamellar body disintegration (to a lesser extent than found in newborn rats and guinea pigs treated with BSO) and decreased amounts of intraalveolar tubular myelin (the precursor of surfactant secreted by the lamellar bodies). This is associated with oxidative destruction of the perilamellar membrane, which contains phosphatidic acid phosphatase and choline phosphotransferase, the key enzymes needed for the synthesis of phosphatidylcholine, the major component of surfactant. GSH deficiency in adult mice is associated with decreased levels of phosphatidylcholine in the lung and in the bronchoalveolar fluid; when ascorbate is given, lamellar body damage does not occur and the levels of phosphatidylcholine increase by about 2-fold (13).

These observations indicate that the lung, which is directly exposed to oxygen, is particularly sensitive to the effects of GSH deficiency. The findings summarized above indicate that an important function of GSH is reduction of dehydroascorbate. In animals that do not synthesize ascorbate, GSH deficiency has more severe and lethal effects, which can be overcome by giving ascorbate.

Ascorbate Deficiency in Guinea Pigs; Sparing of Ascorbate by GSH

It is well known that guinea pigs given an ascorbate-deficient diet develop scurvy and die within 21–24 days. There is a marked loss of weight (after the first week), which is followed by the appearance of characteristic bone changes and hematomas. Treatment of such animals with GSH ester significantly delayed the onset of scurvy; there were no signs of scurvy after 40 days (33). When guinea pigs receiving a scorbutic diet were given GSH ester, the tissue ascorbate levels (as well as the GSH levels) were higher than those of saline-treated controls. The loss of ascorbate was slowed in the presence of higher levels of GSH, another *in vivo* result that supports a role of GSH in the reduction of dehydroascorbate. The findings indicate that GSH, supplied as an ester, spares ascorbate.

GSH-Ascorbate Interrelationships; Animal Models

Ascorbate and GSH have actions in common and can spare each other under appropriate experimental conditions; this redundancy reflects the metabolic importance of such antioxidant activity. An attempt to explain the observations in terms of known reactions is given in Fig. 1. Both ascorbate and GSH can react with hydrogen peroxide and oxygen free radicals (*[O]* in Fig. 1). Although several proteins have been found that catalyze GSH-dependent reduction of dehydroascorbate, a specific dehydroascorbate reductase has not yet been obtained from animal tissues. GSH-dependent reduction of dehydroascorbate is catalyzed by highly purified transhydrogenases from animal tissues (*e.g.* glutaredoxin (24), protein disulfide isomerase (24), and homogeneous transhydrogenase preparations from several animal tissues).[5] GSH-dehydroascorbate reductase activity has thus far not been found in human erythrocytes[5] nor have GSH transhydrogenases apparently been found in mitochondria. Nonenzymatic reduction of dehydroascorbate by GSH, which is rapid (25), may be driven by GSSG reductase in these locations. It is well known that ascorbate is stabilized by thiols, and this is consistent with the *in vivo* finding of decreased tissue ascorbate levels in GSH deficiency. Deficiency of GSH could conceivably lead to inactivation of other systems that may be involved in reduction of dehydroascorbate or to increased metabolic demand for NADPH. Although the *in vivo* data clearly indicate a function of GSH in the reduction of dehydroascorbate, the enzymology involved needs more study. Possibly dehydroascorbate is oriented *in vivo* at sites favorable for reduction by GSH (see prolyl hydroxylase, below). Possibly stable GSH-dehydroascorbate complexes are involved (34). More also needs to be learned about the closely related and important topic of transport of ascorbate and dehydroascorbate across cell membranes. Early studies suggested that dehydroascorbate is a major transport form of ascorbate and that the glucose transporter is involved (see Ref. 35). Neutrophils accumulate ascorbate by a mechanism involving oxidation of extracellular ascorbate to dehydroascorbate, transport of this, and intracellular reduction (36). In an elegant study (37), it was shown that dehydroascorbate (but not ascorbate) is transported into *Xenopus* oocytes expressing mammalian hexose transporters. There seem to be multiple pathways of ascorbate oxidation, reduction, and transport. Ascorbyl radical (semidehydroascorbate) is formed from ascorbate in the β-hydroxylation of dopamine and in other ways; thus, semidehydroascorbate may be formed by interaction of dehydroascorbate and ascorbate. The NADH-dependent semidehydroascorbate reductase system (38–42), not yet fully characterized, catalyzes the one-electron reduction of ascorbyl radical and thus functions in conservation of cellular ascorbate. GSH-dependent reduction of dehydroascorbate is apparently important but not the sole pathway for maintenance of ascorbate.

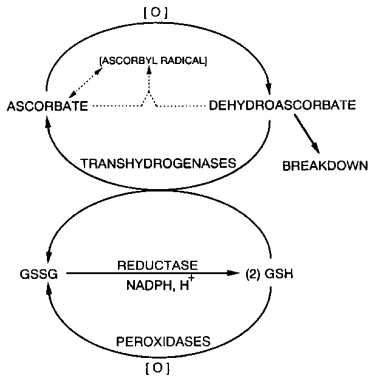

FIG. 1. **Glutathione-ascorbate interrelationships (see the text).** *[O]*, reactive oxygen substances.

Some of the sparing effects of ascorbate on GSH summarized above may be associated with nonenzymatic reactions; this would be consistent with the finding that relatively high doses (*e.g.* 1 mmol/kg/day) of ascorbate are required for *in vivo* effects. Interaction of hydrogen peroxide and ascorbate is enzyme-catalyzed in chloroplasts, cyanobacteria, and soybean nodules (43), and an analogous reaction may occur in animals.

Many *in vitro* studies support the idea that ascorbate can reduce the tocopheroxy radical (see Ref. 44). An elegant experiment in guinea pigs showed that ascorbate does not spare α-tocopherol *in*

[5] E. Szymanska and A. Meister, unpublished data.

TABLE I
Experimental models of GSH deficiency and ascorbate deficiency

Model	Species (Ref.)	Treatment	Result
1	Adult mouse (10,13)	BSO	Tissue damage
		BSO + GSH ester	Protection
		BSO + ascorbate	Protection
2	Newborn rat (4,5)	BSO	Tissue damage; death ~5 days
		BSO + GSH ester	Protection
		BSO + ascorbate	Protection
3	Guinea pig (12)	BSO	Tissue damage; death, ~5 days
		BSO + ascorbate	Protection
4	Guinea pig (33)	Ascorbate-deficient diet	Scurvy; death, ~21 days
		Ascorbate-deficient diet + GSH ester	Scurvy prevented for at least 40 days
		Ascorbate-deficient diet + GSH[a]	Death, ~9 days

[a] Guinea pigs given an ascorbate-deficient diet and GSH itself (3.75 mmol/kg/day) showed hair loss and loss of weight and became moribund after ~9 days. Identical treatment of control animals did not cause toxicity, but similar toxicity was found with higher doses of GSH (7.5 mmol/kg/day). (No toxicity was found with comparable doses of GSH ester.)

vivo (45), but this does not exclude an *in vivo* role of ascorbate, which nevertheless remains unproved. Several *in vitro* studies suggest that the tocopheroxy radical is reduced by a GSH-dependent enzyme (46–51); thus, the tocopheroxy radical (shown by ESR spectroscopy in liver microsomal membranes) was reduced to tocopherol by a heat-labile protein that uses GSH (50). Such a function of GSH, as yet unproved *in vivo*, may be connected with the widespread occurrence of cellular export of GSH (52). Treatment of newborn rats with BSO led to decreased levels of α-tocopherol in the liver[6]; further studies are needed to determine the effects of GSH deficiency on the tocopherols and other membrane components. Possibly GSH functions in maintaining α-tocopherol in two ways, one direct and the other via ascorbate as has been suggested for platelets (53). This idea would be consistent with studies on the "pecking order" of various antioxidants (54).

Although there is significant overlap in the functions of GSH and ascorbate in the destruction of reactive oxygen compounds, GSH has functions that are not served by ascorbate, and ascorbate performs functions that are not efficiently carried out by GSH. There are probably critically essential minimum levels of both ascorbate and of GSH. Some of the animal models that have been examined are summarized in Table I. Treatment with GSH itself of guinea pigs fed a scorbutic diet (model 4) led to death within 9 days without producing signs of scurvy or indeed of other pathology that could be detected by microscopy. The mechanism of this toxic effect of GSH in guinea pigs is not yet clear; possibly it involves extracellular oxidation to form superoxide and peroxide (55). Apparently, ascorbate protects against it (Table I, Footnote *a*). It is generally observed that guinea pigs are more sensitive to various types of stress than are rats and many other animals. Newborn rats are also sensitive, and doses of 5 mmol/kg/day GSH or ascorbate are fatal to them (5). It would not be unexpected to find that superoxide and superoxide dismutase (56) are of significance in GSH-ascorbate interrelationships. Although superoxide seems not to be directly toxic, it can lead to iron-dependent hydroxyl radical formation. An interesting hypothesis about the role of superoxide dismutase led to the suggestion that superoxide might produce oxidative stress by oxidizing GSH (57).

Deficiency of either GSH (not a vitamin) or of ascorbate (a vitamin for guinea pigs, newborn rats, and humans) leads to early mortality, but these conditions reflect quite different manifestations of oxidative stress. Newborn rats and guinea pigs die within 4–6 days when given BSO, whereas guinea pigs deprived of dietary ascorbate show morbidity at 14–17 days and die shortly thereafter. In GSH deficiency, there is rapid development of mitochondrial degeneration with multiorgan failure, whereas in scurvy there is decreased synthesis of collagen and bone associated with oxidative inactivation of prolyl hydroxylase and probably other hydroxylases that require Fe^{2+} (and which are inactivated by oxidation to the Fe^{3+} forms; the same may apply to certain copper-containing enzymes) (58, 59). The bone defects characteristic of scurvy may reflect similar oxidative inactivation of an enzyme needed for the formation of 1,25-dihydroxycholecalciferol (60). Scorbutic guinea pigs continue to synthesize GSH in several tissues, although the tissue levels are lower than normal (33). Notably, the β-subunit of prolyl hydroxylase has GSH-dependent protein disulfide isomerase activity (61). Prolyl hydroxylase (α-subunit), which tends to undergo oxidative inactivation readily, is reactivated by ascorbate (62). GSH-dependent reduction of the dehydroascorbate formed in the reactivation reaction can apparently be catalyzed by the β-subunit (24), suggesting that both ascorbate and GSH contribute to the prolyl hydroxylase reaction. The presence of higher levels of GSH (in the GSH ester-treated guinea pigs) (33) would tend to promote formation of ascorbate from dehydroascorbate at a site (β-subunit) close to the active site of the oxygenase (α-subunit).

Elucidation of the GSH-ascorbate system suggests that some previous studies on the effects of GSH deficiency in rats and mice (and on other animals that can synthesize ascorbate) may need to be re-interpreted because these models of GSH deficiency are complicated by induction of ascorbate synthesis. For example, treatment of rats or mice with BSO might conceivably increase brain ascorbate; ascorbate (but not BSO) is well transported into brain. In this respect, the guinea pig might be a better animal model for studies on GSH deficiency and more reflective of human metabolism. The doses of ascorbate and BSO can probably be adjusted to provide estimates of the GSH and ascorbate minimally needed. (Decreased GSH synthesis may also be accomplished by genetic approaches (see below).) Although large doses of ascorbate can prevent death of BSO-treated guinea pigs, effects of GSH deficiency unrelated to those duplicated by ascorbate may be revealed by further work in which the animals are supplied with sufficient ascorbate to prevent the toxic effects of reactive oxygen substances.

On the "Oxygen" Theory of Disease

There is a growing literature on the toxic effects of oxygen free radicals and reactive oxygen compounds, and it is often suggested that these lead to a wide variety of conditions including ageing, cancer, atherosclerosis, viral infections including AIDS, stroke, myocardial infarction, and arthritis (1).[1] Oxidative stress may not be the fundamental cause of each of these, but it is nevertheless interesting to speculate that a variety of specific pathological and degenerative processes may render cells more susceptible to oxidative damage. Although each of these conditions has oxidative aspects, the specific pathological events and the sequence in which these occur are likely to vary in different diseases and to depend on the cellular components affected (*e.g.* mitochondria, various membranes, cytosolic components) and their turnover rates. More understanding is required about the mechanisms responsible for the detection and disposal of damaged molecules and the repair processes, any of which might be limiting in particular clinical syndromes. Despite the complexities involved, it is possible to obtain disease models by producing alterations in the availability of oxygen and by varying the levels of protective antioxidant substances or enzymes.

It is of interest to further study model systems in which BSO has been applied. The resulting oxidative stress is of endogenous origin, in contrast to that produced by application of various oxidizing

[6] P. McCay, G. Wallis, E. Stole, A. Jain, J. Mårtensson, and A. Meister, unpublished data.

agents, drugs, and radiation. The substantial degree of cellular damage found in GSH deficiency reflects the extensive formation of reactive oxygen intermediates that occurs normally and which is normally opposed by reactions involving GSH. A model of cataract formation in which small doses of BSO are given to newborn rats and mice (or to pregnant animals) has been used (11). Other BSO models that lead to cellular damage to muscle (63), lung (13, 64), jejunum, and colon (65) have been examined. GSH deficiency in newborn rats, which leads to multiorgan failure, has been proposed as a model for evaluation of the efficacy of compounds in preventing oxidative stress (5). In the BSO models, treatment with GSH esters is effective in preventing cellular damage. Since ascorbate has an essential antioxidant function in experimental GSH deficiency (4), it might be expected to be a useful therapy in pathological states in which there is generalized or local GSH deficiency. It may be valuable for treating patients with inborn errors involving defects in GSH synthesis.

Conditions associated with oxidative stress might be ameliorated by therapy that increases cellular GSH levels or the cellular capacity for GSH synthesis, or both. This would be in accord with the established role of GSH as an antioxidant and its function in providing reducing power for maintenance of ascorbate, α-tocopherol, and other cellular constituents. Recent studies suggest that oxidized LDL is involved in the development of atherosclerosis (66), and experimental trials of various antioxidants have been suggested. Therapy designed to increase tissue GSH would be likely to decrease oxidation of LDL (15, 67). There is recent evidence that the GSH status of macrophages in the arterial wall may affect the formation of foam cells and promote elimination of oxidized LDL (68). Thus, the biochemical lesion involved in formation of oxidized LDL may be influenced by the available cellular reducing power. Therapy based on an increase of cellular GSH may also be useful in other conditions associated with oxidative phenomena (see Ref. 15) and in premature infants who may have a deficiency in the cystathionine pathway (69); decreased levels of plasma GSH have been found in pre-term as compared with full-term infants (13). Methods that increase cellular levels of GSH (10) include those based on administration of precursors of substrates for γ-glutamylcysteine synthetase and GSH synthetase, and of GSH derivatives such as esters (8–10).[4] Cellular GSH levels may also be increased by increasing the levels of the synthetases; this has been accomplished in microorganisms (70), and studies on animals are now feasible (see Refs. 71 and 72) and in progress. Genetic approaches to producing a decrease in GSH synthesis are also feasible and would offer an alternative to administration of BSO. It would be interesting to determine the effects of introducing the gene for gulonolactone oxidase (73) into guinea pigs.

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